Using local anaesthetics to block neuronal activity and map specific learning tasks to the mushroom bodies of an insect brain

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Abstract
The formation of a stable olfactory memory requires activity within several brain regions. The honeybee provides a valuable model to map complex olfactory learning tasks onto certain brain areas. To this end, we used injections of the local anaesthetic procaine to reversibly block spike activity in a specific brain region, the mushroom body (MB). We first investigated the physiological effects of procaine on cultured MB neurons from adult honeybee brains. Using the whole-cell configuration of the patch-clamp technique, we show that procaine blocks voltage-gated Na+ and K+ currents in a dose-dependent manner between 0.1 and 10 mM. The effects are reversible within a few minutes of wash. Lidocaine acts similarly, but is less effective at the tested concentrations. We then studied the role of the MBs during reversal learning by blocking the neural activity within these structures by injecting procaine. During reversal learning bees learn to revert their responses to two odors, one rewarded (A+) and one unrewarded (B−), if their contingencies are changed (A− vs B+). Injecting procaine into the MBs impaired reversal learning. Procaine treatment during acquisition left the later retention of the initial learning (A+ vs B−) intact. Similarly, a differential conditioning task involving novel odors (C+ vs D−) was intact under procaine treatment. Our experiments show that local injections of procaine can be used to map learning tasks onto specific regions of the insect brain. We conclude that intact MB activity is required for the acquisition of reversal learning, but not for simple differential learning tasks.

Introduction
Local anaesthetics can be used to locally and reversibly block spike activity within the brain to study how specific brain regions control behaviour. The primary targets of, for example lidocaine and procaine, commonly used anaesthetics, are voltage-sensitive Na+ channels (e.g. Hille, 1966; Bräü et al., 1998; Sheets & Hanck, 2003; Leuwer et al., 2004; for review, see Scholz, 2002). However, they also block other voltage-sensitive channels (e.g. Sugiyama & Muteki, 1994; Komai & McDowell, 2001; Bischoff et al., 2003) as well as ligand-gated ion channels (e.g. Cuevas & Adams, 1994; Nishizawa et al., 2002). Studies of the physiological effects of local anaesthetics on invertebrate spikes or ionic currents are comparably rare, although the first voltage-clamp experiments on the actions of procaine were performed in an invertebrate preparation (Fatt & Katz, 1953; squid: Taylor, 1959; blowflies: Wolbarsht & Hanson, 1965; Washio, 1972; cockroaches: Lapied et al., 2001; crustaceans: Anwyl, 1977; Uusitalo et al., 1995; Theander et al., 1996; C. elegans: Franks et al., 2002). These experiments indicate a similar mode of action in invertebrates and vertebrates.

Most studies of the behavioural effects of anaesthetic treatment of specific brain regions have been performed on mammals (e.g. Bobbot et al., 1996; Daumas et al., 2005; Martin et al., 2006; Teixeira et al., 2006). Previous work by Müller et al. (2003), using systemic injections, showed reversible impairments of motor responses and memory performances in honeybees. Before using local anaesthetics in vivo, however, we provide a detailed analysis of their physiological effects on cultured honeybee neurons where the voltage-sensitive ionic currents are well investigated (e.g. Schäfer et al., 1994; Pelz et al., 1999; Grünewald, 2003; Wüstenberg et al., 2004). For behavioural experiments we used the olfactory conditioning of the proboscis extension reflex (Kuwabara, 1957; Bitterman et al., 1983), which is a well-established paradigm to study the neural control of learning and memory formation in bees. Here, we applied intraneuripilar injections of local anaesthetics to localize acquisition and memory formation using this paradigm. We specifically addressed the role of one brain area, the mushroom bodies (MBs), which has been repeatedly associated with learning and memory capabilities (Menzel et al., 1974; Erber et al., 1980; Menzel, 1999, 2001), and more recently with specific forms of higher-order learning and memory (Giurfa, 2003).

During olfactory conditioning, primary associations between an odor (the conditioned stimulus, CS) and the reward (sucrose solution: the unconditioned stimulus, US) may be formed within the antennal lobes (ALs) and the MBs (Hammer, 1993; Hammer & Menzel, 1998). Conditioned responses (CRs) during acquisition are apparently independent of proper MB function (Mahlun et al., 2002b; Komischke et al., 2005). By contrast, formation, consolidation and retrieval of memory, particularly long-term memory, require functional MBs...
(Menzel et al., 1974; Erber et al., 1980; Cano-Lozano et al., 1996, 2001; Komischke et al., 2005; Locatelli et al., 2005). Because the MBs appear to play only a minor role during simple learning forms like differential conditioning, several researchers argued that the honeybee MBs might rather be important during complex learning behaviour (Menzel & Giurfa, 2001; Giurfa, 2003, 2007; Lachnit et al., 2004). Reversal learning may be regarded as an ambiguous task, as transition between two discrimination phases implies a change in the valence of odors with opposed contingencies. Our hypothesis was that MB activity was necessary during reversal learning. By injecting procaine into the main output regions of the MBs, the α-lobes, we showed that the acquisition of reversal learning was impaired by MB blockade, leaving differential learning unaffected. This indicates that output from the MBs is required for the acquisition of reversal learning in honeybees.

Materials and methods

Electrophysiology

Animals and cell preparation

Adult honeybees (Apis mellifera) were collected from the comb or entrance of hives from the Neurobiology Institute. All electrophysiological experiments were performed in Berlin.

The procedures for dissecting and culturing of Kenyon cells from adult honeybees were similar to those described earlier for pupal cells (Kreissl & Bicker, 1992; Grünewald, 2003). Brains were removed from the head capsule and transferred into a Leibovitz L15 medium (Gibco BRL) supplemented with (in mM): sucrose, 123; glucose, 22.2; fructose, 13.9; proline, 28.7 (500 mOsmol, pH 7.2). The glial sheath was gently removed and the MBs were dissected out of the brains. After incubation (10 min) in a calcium-free saline solution (in mM: NaCl, 130; KCl, 5; MgCl₂, 10; glucose, 25; sucrose, 180; HEPES, 10; pH 7.2), MBs were transferred to L15 medium containing 1 mg/mL collagenase/dispace (30 min). Thereafter, the tissue was rinsed twice with L15 and dissociated by gentle trituration with a 100-μL Eppendorf pipette. Cells were plated in 10-μL samples on Falcon plastic dishes coated with poly-lysine (poly-lysine-L-hydrobromide, MW > 300 kDa Biochrom, Berlin, Germany) and allowed to settle and adhere to the substrate for at least 10 min. Thereafter, the dishes were filled with approximately 2.5 mL of culture medium [13% (v/v) heat-inactivated foetal calf serum (Sigma, St Louis, MO, USA), 1.3% (v/v) yeast hydrolysate (Sigma), 12.5% (w/v) L-15 powder medium (Gibco BRL), 18.9 mM glucose, 11.6 mM fructose, 24.7 mM proline, 93.5 mM sucrose 8 μg/mL gentamycin (from a stock solution of 10 mg/mL, Sigma); adjusted to pH 6.7 with NaOH; 500 mOsmol] and were kept at 26°C in an incubator at high humidity. For electrophysiological measurements, cells were used between culture days 3 and 7. Processes of those cells chosen for recordings did not overlap with neighbouring neurites.

Electrophysiological techniques

Whole-cell gigahm seal recordings were performed at room temperature following the methods described by Hamill et al. (1981). Electrodies were pulled from borosilicate glass capillaries (1.5 mm o.d., 0.8 mm i.d., GB150-8P, Science Products, Hofheim, Germany) with a horizontal puller (DMZ-Universal Puller, Zeitz-Instrumente, Munich, Germany), and had tip resistances between 5 and 10 MΩ in standard external solution (see below). Recordings were made using a HEKA EPC9 amplifier (HEKA-Elektronik, Dr Schulz GmbH, Lamprecht, Germany). Pulse generation, data acquisition and analysis were carried out using PULSE and PULSE-FIT softwares (version 8.53, HEKA-Elektronik) under Windows XP. Currents were low-pass filtered with a four-pole Bessel (~3 dB) filter and sampled at 10–20 kHz. Pipette offset potentials were nulled prior to seal formation; leakage currents were not subtracted. Series resistances ranged between 5 and 20 MΩ, and were compensated at approximately 85%. For data analyses we used IGOR Pro, version 3.15 (Wavemetrics, Lake Oswego, OR, USA).

Solutions

The bath (chamber volume: ~1 mL) was continuously perfused at flow rates of 1–4 mL/min with a standard external solution that consisted of (in mM): NaCl, 130; KCl, 6; MgCl₂, 4; CaCl₂, 5; sucrose, 160; glucose, 25; HEPES-NaOH, 10; pH 6.7, 520 mOsmol/L. To record currents through K⁺ channels, tetrodotoxin (100 nM) was added to the saline to block voltage-gated Na⁺ currents. Some experiments were performed with 50 μM CdCl₂ in the solution to block Ca²⁺ currents. The standard internal solution contained (in mM): KCl, 20; K-glucosone, 87; Na₂-ATP, 3; CaCl₂, 0.2; MgCl₂, 3; K-EGTA, 10; glutathione, 3; GTP-Mg, 0.1; HEPES-bis-tris, 10; sucrose, 120; KF, 40; pH 6.7, 500 mOsmol/L.

To record currents through Na⁺ channels, K⁺ ions in the micropipette solution were replaced by TEA or Cs⁺, Cs-glucosone, TEA-Cl, Cs-EGTA and CsF replaced the corresponding K⁺ salts (in mM: TEA-Cl, 20; Cs-glucosone, 83; Na₂-ATP, 3; CaCl₂, 0.2; MgCl₂, 3; Cs-EGTA, 10; glutathione, 3; Mg-GTP, 0.1; HEPES-bis-tris, 10; sucrose, 120; CsF, 40). All chemicals were purchased from Sigma.

Statistical analyses

Only procaine experiments were statistically evaluated, because we performed very few experiments with lidocaine. To determine concentration effects of procaine on voltage-sensitive ionic currents and to test whether procaine differently blocked currents through K⁺ and Na⁺ channels, various statistical procedures were employed. A three-factor repeated-measure ANOVA was used to test for concentration effects, because each cell was usually tested several times with different procaine concentrations. Differences between procaine effects on Na⁺ and K⁺ currents were tested with an ANOVA. For post hoc comparisons we employed the Scheffé post hoc test. All statistical analyses were performed using STATISTICA (StatSoft, Tulsa, OK, USA).

Behavioural experiments

Animals

All behavioural experiments were performed in Toulouse. Honeybees (Apis mellifera) were caught from hives of the Research Institute on Animal Cognition in the morning of each experimental day, and immobilized by brief cooling on ice. They were then harnessed in individual metal tubes that allowed free movements of their antennae and mouthparts. A small drop of melted bee wax was used to fix the back of the head to the tube to avoid rotational movements during conditioning. Bees were then prepared for intracerebral injections (see below). A window was cut in the head cuticle to give frontal access to the brain. The glands and parts of the tracheae were carefully removed, but the neurilemma was left intact. After placing the piece of cuticle back to its original position, bees were fed with two drops of sucrose solution (50% in water) and left to rest for 3 h in a dark and humid chamber before the experiment started.

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**Conditioning protocols**

A standardized protocol was used for acquisition and retrieval trials, as described recently by Stollhoff et al. (2005). Each acquisition trial lasted 40 s. It consisted of putting a bee from its resting position to the test position into an incoming odourless air flow. An odour stimulus (the CS) was applied 15 s later (duration 4 s) by passing the air flow in a syringe containing a filter paper soaked with 4 µL of odont. Changes from pure air to odont and vice versa were operated automatically using computer-controlled magnetic valves, keeping the flow intensity constant. Presentation of the US started 3 s after odour onset. For this both antennae were touched by a toothpick soaked in 50% sucrose solution to induce proboscis extension and thus ingestion of sugar as the US. Thereafter bees were allowed to lick sucrose solution with their proboscis for 3 s (i.e. about 3 µL), with a 1 s overlap with the CS. Unrewarded CS presentations followed the same timing, except that no sucrose stimulation was applied.

**Pilot experiments.** At the beginning of this study, two pilot experiments were designed to establish the optimal conditions for a behavioural effect of procaine. In both cases, bees were trained to learn a simple association between a CS (limonene) and the sugar reward (A+). The acquisition phase consisted of six trials separated by a 10-min interval. In a first experiment, bees received injections 45 min before the start of conditioning, in both ALs. Independent groups were injected with saline and procaine at one of the following concentrations (w/w in saline, see below): 4%, 10% or 20% (about 148, 370 and 740 mM, respectively). In a second experiment, injections of 20% procaine (in both ALs) were performed at different times before conditioning, i.e. either 15, 30, 45, 60 or 90 min. Saline injections were also performed at all time points in independent groups.

**Reversal learning.** In all experiments on reversal learning, bees were injected in the α-lobes of the MBs. These structures are at the base of the pedunculus and constitute the output region of MBs. Bees were first subject to a differential conditioning with two odorants A and B, one being rewarded with a sucrose solution and the other presented unrewarded (phase 1: A+ vs B−). After 45 min of rest, bees that performed correctly the discrimination in at least one block received an injection of either procaine or saline (see below). Bees were then trained during a second acquisition phase (phase 2) starting 15 min after injections (i.e. 1 h after the end of the initial training). Three different protocols were used during phase 2. (i) In the reversal learning experiment, the same odorants as in phase 1 were presented, but with reversed contingencies: odorant B was thus rewarded and odorant A was presented without reward (A− vs B+). (ii) In the differential conditioning experiment, phase 2 was similar to phase 1, but with two novel odorants (C+ vs D−). (iii) In the extinction experiment, odorants A and B were again presented in phase 2, but without reward (A− vs B−). For all experiments, both phase 1 and 2 consisted of five trials for each odorant (10 trials in total) with 8-min intertrial intervals. The odorants were presented in a pseudo-randomized sequence. During the retention tests (1 h after phase 2) the odorants used for conditioning were presented without reward (CS-only trials). Finally, the unconditioned response (UR) was assayed for each bee. Only bees that responded to the sucrose stimulus were considered for analysis. The odorants used were limonene, eugenol (A and B), 1-heptanal, 1-nonanol (C and D), and were purchased from Sigma-Aldrich (Lyon, France). All experiments were balanced with respect to odour identity.

**Injections**

Procaine was dissolved in a saline that consisted of (in mM): sucrose, 160; glucose, 25; HEPES, 10; MgCl₂, 4; NaCl, 130; KCl, 6; CaCl₂, 2 (pH 6.7). Except for preliminary experiments (see above), the concentration of the procaine solution was 20% (740 mM), and all injections were performed 15 min before the start of phase 2. For behavioural experiments, we rather indicate dilutions (in percentage) instead of concentrations as in patch-clamp experiments (in mM) in order to enable a comparison with the work of Müller et al. (2003). For clarity, the corresponding concentrations in mM have been indicated in the text and figure legends. Each injection procedure took less than 10 min. A volume of 0.5 nL of either procaine solution or saline alone was injected in each α-lobe using a pulled glass capillary (GC 100-10, Harvard Apparatus, Les Ulis, France) connected to a pressure microinjector (IM 300, Narishige, London, UK). The volume of the injected solution was adjusted by injecting saline into a drop of paraffin oil prior to the injection phase and immediately thereafter. Methylene blue (2 mM, Sigma-Aldrich, Lyon, France) was added to the solutions to control for successful injections and correct location of the injection.

**Statistical analysis**

All results are presented as percentages of proboscis extension responses to the odorant applied, ± SEM. Error bars on the graphs represent the 95% confidence limits, as calculated for proportions (Zar, 1999). As our experiments met the criteria required for the application of ANOVA to a dichotomous dependent variable (Lunney, 1970), repeated-measurement ANOVA was used for between-group and within-group comparisons for each acquisition phase. A Wilcoxon test was performed to determine differences between response rates to the CS during specific trials of either phase of acquisition and during the retention test. In the pilot experiments, comparisons of response rates at the last trial were performed using a Mann–Whitney test. All statistical analyses were performed using SPSS 14.0 (SPSS, La Défense, France).

**Results**

**Procaine and lidocaine inhibit voltage-sensitive ionic currents and spike activity**

**Procaine and lidocaine reduce voltage-sensitive ionic currents**

When taken into primary neuron culture, Kenyon cells from adult honeybee brains survive up to 14 days in vitro. We successfully recorded from the neurons between 2 and 12 days in vitro. The adult Kenyon cell shape is similar to that described for pupal cells (Grünewald, 2003). However, only very few cells show neurite outgrowth in the dish. After rupturing the cell membrane, Kenyon cells were voltage-clamped to a holding potential of −80 mV. Depolarizing voltage pulses were then applied to elicit voltage-sensitive ionic currents. Subsequently, we determined the effects of procaine and lidocaine onto the isolated current components.

**Na⁺ currents.** Sodium currents (I₅Na) were isolated by blocking voltage-gated Ca²⁺ and K⁺ currents. Voltage-sensitive Ca²⁺ currents were blocked by adding 50 µM CdCl₂ to the bath solution, and K⁺ currents were blocked by substituting Cs⁺ (133 mM) for K⁺ and adding 20 mM TEA to the pipette solution (Fig. 1). I₅Na activated at voltages more depolarized than −40 mV and peaked between −10 and +4 mV (Fig. 1C). The peak current ranged between −51.4 and −418.4 pA, with a mean amplitude of −195.7 ± 51.4 pA (SEM, N = 27).
Procaine inhibited currents through sodium channels in a concentration-dependent manner between 0.1 and 10 mM ($P<0.05$; $F=8.55$, repeated-measure ANOVA). The effects were completely reversible after 1–3 min of wash (Fig. 1A and B). Procaine applications reduced the mean peak amplitude of the Na$^+$ current to 64.7 ± 2.8% (SEM, $N=29$; Fig. 1E) and 49.7 ± 5.4% (5 mM, $N=25$). At a procaine concentration of 10 mM, the mean Na$^+$ current amplitude reached only 38.9 ± 4.6% of its initial level ($P<0.05$, repeated-measure ANOVA). Significant differences between different concentrations (Scheffe post hoc test) are indicated by different letters. Currents are normalized to $I_{\text{max}}$ measured at a command potential of –10 mV during the last activation protocol before procaine application. Plotted are the mean relative currents (± SEM) before and during anaesthetic (drug) applications and during wash. (F) Lidocaine blocked Na$^+$ currents, but appears less effective than procaine (no block at 1 mM). However, note that the number of experiments during these experiments was smaller, statistical differences were therefore not tested.

**K$^+$ currents.** Honeybee neurons express various voltage-sensitive K$^+$ currents. These currents were described in detail on cells cultured from pupal brains (Schäfer 	extit{et al.}, 1994; Pelz 	extit{et al.}, 1999; Grünewald, 2003; Wüstenberg 	extit{et al.}, 2004). Adult Kenyon cells show very similar K$^+$ currents with respect to steady-state activation as well as the overall dynamics (Fig. 2). For comparison of the procaine effects we measured the current amplitudes at two different time points during the depolarizing test pulse. Firstly, the peak current reflects the transient, inactivating K$^+$ current that consists mainly of an A-type K$^+$ current (Pelz 	extit{et al.}, 1999). Secondly, the sustained current, measured at the end of the 100-ms test pulse, comprises mainly the delayed rectifier K$^+$ current. The mean peak amplitude of the transient current at a holding potential of +50 mV was 1630.5 ± 121.2 pA (SEM, $N=22$; range 587.0–2982.2 pA). The current amplitude of the sustained current component was 364.0 ± 34.1 pA (SEM, $N=22$; range 144.6–693.4 pA). The ratio of peak (transient) vs sustained K$^+$ current was 4.87 ± 0.37. This is higher than the ratio of Kenyon cells from pupal brain tissue obtained in an earlier study (2.8 ± 0.25; $N=10$, Grünewald, 2003).
Procaine reversibly blocked voltage-sensitive \( K^+ \) currents (Fig. 2A). At a concentration of 1 mM it reduced the peak amplitude to \( 87.1 \pm 2.3\% \) and the sustained \( K^+ \) current component to \( 77.1 \pm 3.2\% \) (\( N = 25; V_{\text{hold}} = 50 \text{ mV}; \text{Fig. 2D} \)). The procaine block was dose-dependent (\( P < 0.00001, F = 46.68, \text{d.f.} = 16, \text{repeated-measure ANOVA} \)). The maximum block (at 10 mM) ranged between 65 and 33.7\%, with a mean of 51.6 \( \pm \) 2.2\% of the original current (measured at the peak, \( N = 18; \text{Fig. 2D} \)). Two cells showed a block of more than 60\%, and two cells of less than 40\%. Concentrations higher than 10 mM were not tested due to seal instabilities (see above).
Procaine affected the transient and the sustained K+ current equally (Fig. 2B and D; \(P = 0.25, F = 1.31, \text{d.f.} = 201, \text{ANOV})\). It reduced the current amplitude and did not alter the current kinetics (Fig. 2C).

The voltage-sensitive Na+ current was more sensitive to procaine than the K+ currents at a concentration of 1 mM (\(P < 0.01, \text{Scheffe test}, N = 25\)). No differences were detected for the other procaine concentrations.

**Lidocaine.** Earlier studies (Müller et al., 2003) used lidocaine for behavioural experiments to prevent neural activity in honeybees. We wanted to test whether lidocaine was similarly effective than procaine. Therefore, and because we needed to evaluate which drug was best suited for intraneuropil injections, we also tested the effect of lidocaine on a few neurons. Overall we observed similar effects as for procaine. However, lidocaine appeared to be a less potent Na+ channel blocker than procaine. At a concentration of 1 mM no reduction of the peak Na+ current amplitude was observed (99.4 ± 5.8%, SEM, \(N = 4, \text{Fig. 1F}\)). At higher concentrations the blockade was dose dependent with 70.2 ± 15.6% at 5 mM (\(N = 5\)) and 56.6 ± 12.4% at 10 mM (\(N = 4\)). Lidocaine also inhibited voltage-sensitive K+ currents (Fig. 2D). The lidocaine effects were dose dependent and reduced the peak current amplitude between 64.9 ± 5.6% at 1 mM (\(N = 5\)) and 24.1 ± 3.1% at 10 mM (\(N = 4\)). All lidocaine-induced blockades were completely reversible after a few minutes of wash. Thus, it appears that lidocaine inhibits K+ currents more potently than Na+ currents. We used procaine for injections during the behavioural experiments, because it is more efficiently blocking Na+ currents and it appears to be less toxic than lidocaine (Müller et al., 2003).

**Procaine blocks action potentials in adult Kenyon cells in vitro**

Upon switching the amplifier into the current-clamp mode, a constant current was injected to keep the cell membrane at about −80 mV. When the holding current was removed the cells maintained a membrane potential that varied considerably between −45 and −65 mV. Under these conditions Kenyon cells do not generate spontaneous action potentials, as was similarly shown for pupal cells (Wüstenberg et al., 2004). About 26% of the recorded Kenyon cells (12 of 45 cells) responded to a depolarizing current injection (100 ms −1 s) by firing at least one action potential. The remaining cells failed to generate action potentials. Three cells out of 45 cells spiked repetitively when depolarized with a 1-s, constant-current pulse (Fig. 3A), whereas the remaining cells fired only a single spike to a suprathreshold depolarization. Action potentials were normally overshooting, but otherwise varied considerably in amplitude and duration among different cells. Even within a given cell the spike shape changed during the time course of the recording.

The spike activity was blocked by 2% procaine (corresponding to a concentration of approximately 74 mM) applied to the bath perfusion (\(N = 5\); Fig. 3A and B). Lower concentrations of procaine (0.2–1 mM) did not affect spiking in cultured Kenyon cells. Upon switching to the voltage-clamp mode of the amplifier, we determined the membrane currents of the cell presented in Fig. 3. At a concentration of 74 mM procaine reversibly inhibited the inward current almost completely (Fig. 3B). In addition, procaine also drastically reduced the amplitude of the total outward current. At 5–10 mM procaine the spike duration was substantially prolonged (\(N = 6\); data not shown). The effects of procaine on spikes were reversible within 60 s of wash.

After establishing the physiological efficiency of procaine and lidocaine in vitro, we wanted to test the effect of local anaesthesia of MB α-lobes on reversal learning. Before starting these experiments, we first performed two pilot experiments designed to determine the dose and time of injection in order to block neural activity rapidly enough and during the complete duration of the reversal experiment.

**Local injections of procaine induce dose-dependent and stable behavioural effects**

We first examined the learning performance of bees that had received a bilateral injection of saline or procaine (4%, 10% or 20% in

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**Fig. 3.** Procaine inhibits spike activity. Cultured Kenyon cells generate overshooting action potentials upon injecting depolarizing current pulses (+14 pA in A, 13 pA in B). At about 74 mM (≈2%) procaine added to the external saline spikes are blocked (A). The dashed line indicates a membrane potential of 0 mV. (B) The voltage-sensitive ionic currents of the same cell measured under voltage-clamp. Outward as well as inward currents are reduced. Both the effects on spikes and ionic currents are reversed after 2 min of wash (lower traces in A and B).
saline – corresponding to about 148, 370 and 740 mM, respectively) into the ALs. We targeted this structure because of its well-known implication in olfactory processing, so that we expected olfactory conditioning to be impaired during procaine-induced blockade of neural activity in vivo. The conditioning protocol lasted 60 min, and injections were performed 45 min before it started, so that we compared performances in the last trial at a time that corresponds to 105 min post-injection. This delay was chosen in comparable order to the required duration of procaine action during the next experiments (acquisition or extinction phases of 80 min). As shown in Fig. 4A, procaine-injected bees exhibited a dose-dependent decrease of learning performance as compared with the control group. Relative to control levels (100%), the rates of CRs were reduced after injection of 4% (CR, 72.9%), 10% (CR, 53.7%) and 20% (CR, 47.0%). This dose-dependent learning impairment was significant for the highest dose (20%), although at 10% the effect was marginally significant (Mann–Whitney test: for 10%; \( P = 0.068 \); for 20%; \( P < 0.01 \)). We thus selected 20% as a working dilution for the next experiments.

Once the efficient dose was selected, we varied the delay between injection and conditioning, from 15 min to 90 min (bilateral injection into both ALs; Fig. 4B). After a six-trial conditioning identical to that used in the previous experiment, all groups of procaine-treated bees showed a significant decrease in learning, compared with bees treated with saline at a similar time (\( P < 0.05 \) in all cases, Mann–Whitney). This decrease varied from 37.1% to 42.9% of control bees’ rate of CRs (\( P < 0.05 \) in all cases). However, we found no clear effect of the delay among the values tested, thus we selected the shortest one (15 min before conditioning). For this value, the effect on behaviour was maintained over more than 1 h (delay between injection and last conditioning trial: 45 min). Because reversal learning would consist of two successive acquisition phases (80 min each), separated by a 1-h period during which injections had to be performed (see Materials and methods), we decided to inject 15 min before the second (reversal) phase began. This would ensure to leave consolidation after phase 1 undisturbed for 45 min and to affect neural activity during the whole duration of phase 2.

**Injections of procaine into the \( \alpha \)-lobes impair acquisition of reversal learning**

During this and all following experiments, bees were trained to discriminate two odours A and B during phase 1. One was rewarded (A+) and the other not (B–). The response probability to A+ was higher than to B– during acquisition (Figs 5–8). At the end of the first phase of acquisition (i.e. before injection of procaine), the proportions of CRs to A+ and B– were about 80% and 10%, respectively (\( P < 0.005 \) in both groups, Fig. 5).

One hour later all bees were trained the reversed differential rule, i.e. A– vs B+. Injections of procaine into both \( \alpha \)-lobes differentially affected the acquisition curves during the second phase, as indicated by a significant effect of treatment on responses (\( F_{1,162} = 4.336, \ P < 0.05 \)), as well as a significant treatment–blocks interaction (\( F_{1,162} = 6.296, \ P < 0.005 \); repeated-measurement ANOVA: treatment \( \times \) block \( \times \) CS). This difference could be attributed specifically to the treatment, as no difference in acquisition was found between the two groups before injection, during phase 1, like in all the other experiments. It should be noted that, in our conditions, procaine injections affected neither survival nor the probability of the UR (\( P > 0.05 \) in both cases, Mann–Whitney).

As shown in Fig. 5, saline-injected bees were able to learn the reversed contingencies. They responded significantly more to odour B than to A during the last trial of this second training phase (\( P < 0.001 \), Wilcoxon). By contrast, bees that received procaine injections were not able to reverse the rule, because they did not respond differently to both odorants. Their acquisition curve showed an increased response probability to B+. However, responses to A– did not decrease and the bees did not show significant differences between odour responses to A vs B at the last trial of the reversal training phase. Similarly, they responded equally to odours A and B during the retention tests (1 h later), while saline-injected bees responded more often to odour B (\( P < 0.005 \), Wilcoxon). Thus, \( \alpha \)-lobe blockade by procaine impaired reversal learning.

**Reversal learning impairment after MB blockade is not due to impaired retrieval**

As observed in Fig. 5, procaine injections resulted in a reduction of the initial level of response to A in the first trial of phase 2 for this odorant, compared with the level achieved at the end of phase 1 (\( \sim 40\% \) and 80%, respectively). This decay in retention level to the initially conditioned odour A after procaine administration is in general agreement with a role of the \( \alpha \)-lobes during the recall of an...
odour memory (Cano-Lozano et al., 2001; Menzel, 2001; Locatelli et al., 2005). However, this might explain why procaine-treated bees could not perform reversal learning: because they started phase 2 with a low level of responses to A, they might not have reduced sufficiently their response rates to attain a level lower than that observed in response to B at the end of phase 2. Thus, we asked whether this impaired retrieval could be the actual cause of the absence of reversal by looking specifically at the performance of bees with intact retrieval, i.e. those that did respond to A at the beginning of phase 2. Hence, we analysed again our data, considering only the subgroup of bees that responded to A at the beginning of phase 2. However, this might explain why procaine-treated bees could not perform reversal learning: because they started phase 2 with a low level of responses to A, they might not have reduced sufficiently their response rates to attain a level lower than that observed in response to B at the end of phase 2. Thus, we asked whether this impaired retrieval could be the actual cause of the absence of reversal by looking specifically at the performance of bees with intact retrieval, i.e. those that did respond to A at the beginning of phase 2. Hence, we analysed again our data, considering only the subgroup of bees that responded to A at the beginning of phase 2 (respectively, 67% and 63% of saline- and procaine-treated bees). The corresponding learning curves (Fig. 6A) clearly show that, in this subgroup of bees, reversal learning was impaired after procaine treatment (P > 0.05), but not after saline treatment. A repeated-measurement ANOVA on all responses during phase 2 (extinction phase) revealed that saline- and procaine-treated groups behaved similarly (treatment effect: F1,105 = 7.361, P < 0.01), and that this difference depended on the CS presented (treatment–CS interaction: F1,105 = 4.839, P < 0.05). Saline-treated bees exhibited a significant decrease in their response rates to A when it was no longer rewarded (extinction of a previous acquisition memory, Stollhoff et al., 2005; F1,52 = 3.937, P < 0.01, ANOVA). As expected, responses to B– were very low and did not change. By contrast, procaine-injected bees maintained a constantly increased level of proboscis extension responses (PER) to A throughout all trials. As a result, both groups reached similar response levels to A at the end of phase 2 (group effect for trial 5: F1,105 = 1.08, P > 0.05).

This result may imply a blockade of extinction by procaine. It may, however, also be the consequence of an impaired retrieval as, like in experiment 1, the initial response level was significantly lower in procaine-treated bees (treatment effect on trial 1: F1,105 = 14.28, P < 0.005, ANOVA). Then, extinction may not occur in bees that failed to recall the initial association of odour A with the reward. Thus, we calculated the response rates of both groups during the extinction phase required for A to extinguish, while the response rate to the formerly non-rewarded stimulus (A) should increase. Thus, a possibility is that the impairment of reversal learning in procaine-injected animals might be due to a failure in extinction learning for A. Because the responses of procaine-treated bees to A did not diminish when no longer rewarded (Fig. 5: phase 2), we tested whether extinction was impaired in these animals. For this, we repeatedly presented unrewarded odours A and B to individual bees during phase 2 (A– vs B–, Fig. 7). A first repeated-measurement ANOVA on all responses during phase 2 (extinction phase) revealed that saline- and procaine-treated groups behaved differently (treatment effect: F1,105 = 7.361, P < 0.01), and that this difference depended on the CS presented (treatment–CS interaction: F1,105 = 4.839, P < 0.05). Saline-treated bees exhibited a significant decrease in their response rates to A when it was no longer rewarded (extinction of a previous acquisition memory, Stollhoff et al., 2005; F1,52 = 3.937, P < 0.01, ANOVA). As expected, responses to B– were very low and did not change. By contrast, procaine-injected bees maintained a constantly increased level of proboscis extension responses (PER) to A throughout all trials. As a result, both groups reached similar response levels to A at the end of phase 2 (group effect for trial 5: F1,105 = 1.08, P > 0.05).

Blocking the α-lobes does not impair extinction

Reversal learning requires that the CR to the previously rewarded stimulus (B) should extinguish, while the response rate to the formerly non-rewarded stimulus (A) should increase. Thus, a possibility is that the impairment of reversal learning in procaine-injected animals might be due to a failure in extinction learning for A. Because the responses of procaine-treated bees to A did not diminish when no longer rewarded (Fig. 5: phase 2), we tested whether extinction was impaired in these animals. For this, we repeatedly presented unrewarded odours A and B to individual bees during phase 2 (A– vs B–, Fig. 7). A first repeated-measurement ANOVA on all responses during phase 2 (extinction phase) revealed that saline- and procaine-treated groups behaved differently (treatment effect: F1,105 = 7.361, P < 0.01), and that this difference depended on the CS presented (treatment–CS interaction: F1,105 = 4.839, P < 0.05). Saline-treated bees exhibited a significant decrease in their response rates to A when it was no longer rewarded (extinction of a previous acquisition memory, Stollhoff et al., 2005; F1,52 = 3.937, P < 0.01, ANOVA). As expected, responses to B– were very low and did not change. By contrast, procaine-injected bees maintained a constantly increased level of proboscis extension responses (PER) to A throughout all trials. As a result, both groups reached similar response levels to A at the end of phase 2 (group effect for trial 5: F1,105 = 1.08, P > 0.05).

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Fig. 5. Reversal learning after blocking alpha lobes. Percentage of proboscis extension response (PER) evoked by odorants A (filled bars) and B (open bars). In phase 1 of acquisition, bees from both experimental groups were untreated and had to learn to discriminate between a rewarded odorant (A+) and an unrewarded one (B–). After injections of either saline (above) or procaine (below), phase 2 took place, during which the reversed rule (A–, B+) had to be acquired. A retention test was applied 1 h after the end of acquisition: bees were presented successively with both odorants without reward. **P < 0.01; ***P < 0.005. n = 86 (saline) and 79 (procaine).
phases, considering only those bees that responded to A with a CR during the first extinction trial as we did previously for reversal learning (Fig. 6B). In these bees, the response levels to A decreased along the trials in both groups (block effect; saline: $F_{1,40} = 8.224$, $P < 0.005$; procaine: $F_{1,23} = 4.052$, $P < 0.01$), reaching almost identical levels in block 5 (saline: 63.4%, $n = 41$; procaine: 62.5%, $n = 24$). Thus, whenever retrieval is not affected by procaine, extinction occurs normally. This result was again observed in the retention scores. It should be noted that bees still responded more often to A than to B ($P < 0.005$ for both groups, Wilcoxon test), suggesting that more trials may be necessary for completing extinction.

**Acquisition of differential conditioning remains unaffected by α-lobe blockade**

Bilateral procaine injections may produce a general learning deficit such that the impairment observed for reversal learning may be unspecific, rather than due to the particular learning task employed. To test whether procaine-injected bees are still able to learn an odour, we performed a discriminatory learning task during the second acquisition phase. After injections, bees had to solve a simple differential conditioning task, similar to that learned in phase 1, but using two new odors (C+ vs D–). This control is appropriate as it presents bees with two discrimination problems (phase 1: A+ vs B–; phase 2: C+ vs D–) as in reversal learning, but with the crucial difference of having eliminated the ambiguity in stimulus valence underlying phase transition in reversal learning ($A^{+} \rightarrow B^{−}$ and $B^{+} \rightarrow A^{−}$). In other words, the new discrimination learning proposed to bees was an elemental task for which MBs may not be required. In such conditions, no effect of treatment was observed, neither during acquisition in phase 2 nor during the retention test (Fig. 8). A treatment × blocks × CS repeated-measurement ANOVA revealed that treatment had no significant effect nor interaction with either of the two other factors, thus indicating that procaine injections did not affect the animals’ performance. This was confirmed by an ANOVA for trial 5: animals from both groups were equally able to learn to respond to C but not to D (CS effect: $F_{1,97} = 66.69$, $P < 0.01$; treatment–CS interaction: $F_{1,97} = 0.03$, $P > 0.05$). They also retained this new association for at least 1 h ($P < 0.005$ in both cases, Wilcoxon). These results thus show that blocking neural activity within the α-lobes did not abolish the ability to learn odours in a discriminatory classical conditioning task. Furthermore, procaine-injected bees, like saline-injected bees, also retained their memory for the initial acquisition phase (phase 1, A+/B–; Fig. 8). They responded significantly more to
odour A than to odour B during the retention tests ($P < 0.005$ in both cases, Wilcoxon). The effect of procaine injection in the MBs was therefore specific for reversal learning and not general to any kind of olfactory discrimination.

**Discussion**

To map specific behavioural tasks to particular regions within the vertebrate brain, local anaesthetics have been successfully used as general blockers of neuronal activity in a space- and time-dependent manner (e.g. Griffin & Berry, 2004; Keller et al., 2004; Winters & Bussey, 2005; Teixeira et al., 2006). We used here for the first time a combined physiological and behavioural approach to show that local anaesthetics inhibit spiking and voltage-sensitive ionic currents also in insect neurons, and that procaine can be used to reversibly block neural activity within the insect brain. Lidocaine and procaine, among the most commonly used in mammals, have been recently shown to impair basic forms of olfactory learning and motor responses in bees (Müller et al., 2003). In that study, however, the local anaesthetics were applied through systemic injections in the thorax, i.e. without specifically targeting the brain and without knowing whether the drugs affect neuronal spiking in honeybee neurons.

Here we focused on the effect of these drugs on adult MB neurons. These same neurons were targeted *in vivo* in order to inactivate the function of MBs during several olfactory learning tasks. Under such conditions, we show that bees cannot perform reversal learning, while they still retain the abilities for differential conditioning and extinction. Thus, using the two complementary approaches of behavioural analysis and patch-clamp, we have characterized these anaesthetics as valuable tools to locally block neuronal activity and to trace down specific memory traces in an insect brain.

**Procaine and lidocaine affect insect neuronal ion channels**

While general anaesthetics were frequently tested on insect preparations (Kirschfeld & Bajerrogowski, 1987; Kirschfeld, 1987, 1988; Rajaram & Nash, 2004; van Swinderen, 2006), local anaesthetics have been rarely used. Our patch-clamp analysis on cultured Kenyon cells (MB neurons) has revealed that lidocaine and procaine are efficient blockers of neuronal spiking activity in insects, like in mammals. Our recordings of sodium and potassium currents showed that both were affected by the drugs in a dose-dependent manner, like in mammals (Scholz, 2002). However, our data suggest some possible differences in their respective specificities of action, as procaine appeared to be more efficient to reduce sodium channels, while lidocaine seemed more efficient on potassium currents. Procaine inhibited currents through the voltage-sensitive Na$^+$ channel more efficiently than through K$^+$ channels. The transient and sustained K$^+$ current components were equally sensitive to procaine. Voltage-gated sodium and potassium channels are among the main targets of both drugs, as indicated by previous studies (Scholz, 2002). Thus, although we have not tested other possible molecular targets identified in some vertebrate preparations [e.g. calcium channels, nicotinic or N-methyl-D-aspartate (NMDA) receptors; Sugiyama & Muteki, 2006].

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**Fig. 7.** Extinction after blocking alpha lobes. Percentage of proboscis extension response (PER) evoked by odorants A (filled bars) and B (open bars). Phase 1 of acquisition was identical to that in previous experiments (differential conditioning: $A^+ \text{ vs } B^-$). In phase 2, saline- and procaine-injected bees were submitted to an extinction protocol ($A^– \text{ vs } B^–$). As before, a retention test was applied 1 h after the end of acquisition. $n = 53$ in both groups. The levels of significance indicated for phase 2 correspond to the decrease of response levels to A across trials. n.s., non-significant; **$P < 0.01$; *** $P < 0.005$. 

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*European Journal of Neuroscience, 26*, 3193–3206
1994; Komai & McDowell, 2001; Bischoff et al., 2003], our results argue in favour of a conserved mode of action of procaine and lidocaine across phyla.

The binding site for local anaesthetics resides within the channel pore (Hille, 2001). Thus, local anaesthetics may act in a use-dependent way as open channel blockers (lipophilic pathway). In addition, a use-independent, hydrophilic block of the closed channel state exists. Our data suggest that insect channels do not require activation prior to block. We always observed maximum block during the first depolarizing voltage pulses. Furthermore, we could not observe that procaine altered the gating kinetics of either the Na⁺ or the K⁺ currents (neither activation nor inactivation). Therefore, procaine appears to reduce the channel conductance via a hydrophilic pathway in insect neurons. More detailed biophysical experiments are required before we understand the mode of action of local anaesthetics on insect ion channels.

Output from the MBs is required during reversal learning

MBs are important centres for learning and memory in the insect brain (e.g. Menzel, 2001; Heisenberg, 2003). In bees they receive input from different sensory modalities, in particular from olfactory, visual and gustatory sensory neuropils (for review, see Mobbs, 1982; Strausfeld et al., 1998). Associations between an odorant and a sugar reward can be formed within the MBs during classical olfactory conditioning (Menzel, 2001). However, proper MB functioning is not an absolute requirement for learning, as bees can learn elemental tasks in which stimuli are unambiguously associated with reward or with its absence (Giurfa, 2003), even with absent or severely atrophied MBs (Malun et al., 2002b; Komischke et al., 2005) – contrarily to flies (Heisenberg et al., 1985; deBelle & Heisenberg, 1994; Dubnau et al., 2001; Schwaerzel et al., 2003). Nevertheless, this conclusion does not extend to all forms of olfactory learning tested. Rather, it seems as if MBs were specifically required for some learning tasks only (Komischke et al., 2005), while dispensable in others. What is the specificity of such tasks? One possibility is that MB-dependent tasks include several different stimuli, such as several odours (Komischke et al., 2005).

Else, MB activity may be required to solve conflicts between contradictory CS–US associations. Examples of the latter are opposite association rules processed by each brain hemisphere (side-specific conditioning, Sandoz & Menzel, 2001), or alternative presence or absence of reward associated with the same CS, depending whether it is presented alone or in a mixture (configural learning, Komischke et al., 2003, 2005; Giurfa, 2003; Lachnit et al., 2004). If this was the case, then we could predict that MBs would be necessary for other olfactory tasks involving conflict resolution. Reversal learning belongs to this category, as it involves the sequential processing of contradictory information about associations between CS and US. It was previously shown that intact honeybees are able to perform this task (Komischke et al., 2002).

Here we have shown that injecting procaine into the α-lobes (one of the MB output pathways) profoundly affected the animals’ perfor-

Fig. 8. Differential conditioning after blocking alpha lobes. Percentage of proboscis extension response (PER) evoked by limonene and eugenol (during phase 1, before treatment), and 1-heptanal and 1-nonanol (during phase 2, after treatment) of acquisition. In both phases, one odorant was rewarded while the other was not (A+ vs B, then C vs D). Memory was measured at 1 h by recording the responses to all four unrewarded odorants. **p < 0.01; ***p < 0.005; n = 49 in each group.
mance in this task. Acquisition of reversed CS–US associations was completely impaired. Successful reversal learning implies that two processes take place: (i) extinction, i.e. reduction of the response probability to the initially rewarded odorant (A+, then A–); and (ii) increase of the response probability to the newly rewarded stimulus (B–, then B+). Because the response rates to B+ increase during reversal learning and because discriminatory learning under procaine is intact, we show that odours can be learned as prediction signals to a reward. Furthermore, extinction was not completely impaired by blocking MB function. Rather, some bees failed to remember the initial A/sugar association shortly after injection, and thus could not show extinction. Thus, procaine injections partially impaired retrieval of the learned odour, in accordance with previous studies showing the importance of intact output from the MBs for retrieval (Cano-Lozano et al., 2001). As a consequence, they failed to extinguish their responses to A+, as shown by the drop in the response rate between the last A+ trial during phase 1 and the first A– trial of phase 2. This partial retrieval failure is probably not due to an impaired consolidation because, after differential conditioning, retrieval of the initial association rule (A+, B–) was intact after more than 2.5 h post-injection (see Fig. 8).

Importantly, this retrieval failure is unlikely to explain the reversal learning impairment. Indeed, bees that do not remember phase 1 should perform phase 2 more easily, which would then be similar to a normal differential conditioning task. Besides, those bees with intact retrieval did not display a better performance. Thus, blocking α-lobes leads to an impairment of reversal learning acquisition, which is dissociable from the impairment of retrieval.

Reversible and local effects of intracerebral procaine injections

The finding that retrieval of the initial associative rule was intact 2.5 h after injection indicates that the action of procaine was reversible within 2.5 h. Our pilot experiments showed the effect of injecting the same dose of procaine into the ALs to last at least 90 min. Thus, we conclude that: (i) procaine remains effective on Kenyon cells during the whole second phase of acquisition; (ii) procaine is progressively washed out during the 1 h consolidation phase, i.e. between ~1.5 and 2.5 h post-injection; (iii) the procaine effects are completely reversible. This reversibility is also suggested by the analysis of individual responses curves, which showed that some individuals that failed to respond according to the initial rule at the first trial of phase 2 succeeded in subsequent trials (data not shown). Hence, while some bees correctly increased their response probability to the newly rewarded stimulus, others actually increased it,

A new role for MBs in olfactory learning

Because retrieval seems to be only transiently impaired, a memory of the initial association rule is again accessible in later trials of phase 2 in many individuals. Thus, retrieval impairment alone cannot explain the failure to reverse the rule. Indeed, complete blockade of retrieval should facilitate learning of the new rule, as the memory of the first phase (A+ vs B–) would not interfere with reversal learning. In such a case, procaine-treated bees should behave exactly the same way in the two protocols used, i.e. reversal learning and differential conditioning (control), which is obviously not the case. Thus, other processes that are controlled by MB function must be required for reversal learning, which are impaired by procaine.

First, it is noteworthy that extinction seems to take place normally without functional MBs. This is in contrast with the situation in Drosophila (Schwaerzel et al., 2002). This discrepancy may be a consequence of the use of different networks for learning processes in these species. Because CS–US associations may form outside the ALs in bees, the neural processes of ‘re-learning’ that are believed to underlie extinction (Myers & Davis, 2002; Stollhoff et al., 2005) may take place outside the MBs, in contrast to flies. Second, during our differential conditioning experiment four odours were involved, while reversal learning involves only two odours. Thus, the efficiency of procaine-treated bees to realize the former but not the latter strongly argues against the number of stimuli as the critical parameter for MB requirement in olfactory associative learning, as proposed earlier (Komischke et al., 2005). Another critical difference is that, during differential conditioning, processing the new CS–US associations did not generate any contradiction with those learnt before. Thus, these results suggest a possible role for MBs for the resolution of conflicting information. In honeybees, the question of the respective roles of MBs
and ALs in olfactory learning has been raised since early experiments showed that several memory traces of a given CS–US association could be formed in both structures (Hammer & Menzel, 1998; see Menzel, 2001), but that normal AL function was sufficient to form and consolidate the trace (Malun et al., 2002b; Komischke et al., 2005). This situation contrasts with those found in fruitflies (Heisenberg et al., 1985; de Belle & Heisenberg, 1994; Dubnau et al., 2001) and crickets (Scotto-Lomassese et al., 2003), where ‘simple’ associative learning such as the differential conditioning task used here as a control cannot be learned without normal MB activity. Thus, the present results suggest that in the honeybee, MBs might be necessary during acquisition of ‘complex’ tasks, i.e. those requiring the resolution of contradictory CS–US associations. This functional recruitment of additional areas in more demanding learning tasks reminds several observations on memory consolidation after fear conditioning in rodents. While not required for the processing of associative components of fear conditioning (mostly done in the amygdala), the hippocampus appears to be selectively involved in consolidation and retrieval of the contextual components (Corcoran & Maren, 2001, 2004; Bast et al., 2003; Malin & McGaugh, 2006).

From our hypothesis it can be predicted that other learning tasks involving conflict resolution between multiple CS–US associations are MB dependent (Giurfa, 2003, 2007). This has already been hypothesized for side-specific conditioning, in which the two halves of the brain must deal with different learning rules (Komischke et al., 2003). From this perspective, MBs would play more complex and diverse functions in olfactory learning than previously expected. In particular, they may compute a comparison between conflicting information in order to lead to an appropriate motor output. This is supported by the indication from our data that some memory trace of the first rule (A+ B−) must be normally present in the MBs when acquisition of the new rule (A− B+) starts. Optophysiological recordings have shown learning-induced plasticity in odorant representations in the MBs as early as 10 min after conditioning (Faber & Menzel, 2001). It is thus conceivable that reversal learning involves changes in odorant coding by MB neurons, leading to form different memory traces (e.g. of A+ B−, C− D+) or to eliminate inaccurate ones whenever conflict arrives (i.e. A+, B− in reversal learning). Further experiments will provide insights into the mechanisms underlying conflict resolution in these neural circuits.

Supplementary material
The following supplementary material may be found on http://www.blackwell-synergy.com
Fig. S1. Fluorescent staining of α-lobes with sulphorodamine B, showing that diffusion outside the lobes is limited.

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Abbreviations
ALs, antennal lobes; CR, conditioned response; CS, conditioned stimulus; MBs, mushroom bodies; PER, proboscis extension response; US, unconditioned response; US, unconditioned stimulus.

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